Amendments to the Specification

Please insert the following sentence before the first sentence of the application at page 1:

-- This application is a divisional of Patent Application Serial No. 09/120, 044 filed on July 21, 1998, which claims benefit of Provisional Applications Serial No. 60/053,306 filed on July 21, 1997 and Serial No. 60/073,456 filed on February 2, 1998.- -

On page 8, lns. 2-3, please amend the first paragraph as follows:

FIG. 1A and FIG. 1B: Wild-type nucleic acid sequence of type 14 pneumolysin (SEQ ID NO:1).

On page 8, lns. 4-14, please amend the second paragraph as follows:

FIG. 2A and FIG. 2B: Non-limiting nucleic acid variations of type 14 pneumolysin (SEQ ID NO:2). The residue position followed by examples of nucleic acid substitutions that attenuate hemolytic activity are: 181, C; 443, A; 583, A or G. The residue position followed by examples of nucleic acid substitutions not observed to attenuate hemolytic activity are: 50, G; 54, T; 98, C; 122, G; 134, C; 137, C; 187, T; 196, T; 248, C; 276, C; 302, C; 305, G; 351, T; 380, A; 382, C; 459, C; 514, G; 558, C; 566, G; 717, A; 764, G; 770, G; 1038, T; 1138, A; 1212, A; 1296, T; 1386, G; 1395, A.

On page 8, lns. 15-16, please amend the third paragraph as follows:

FIG. 3A and FIG. 3B: Amino acid sequence of type 14 pneumolysin (SEQ ID NO:3).

On page 8, lns. 17-27, please amend the fourth paragraph as follows:

FIG. 4A and FIG. 4B: Non-limiting amino acid variations of type 14 pneumolysin (SEQ ID NO:4). The residue position followed by examples of amino acid substitutions that attenuate hemolytic activity are: 61, Pro; 148, Lys; 195, Ile or Val; 243, Arg, Val, Glu, or Ser; 286, Asp; 446, Ser. The residue position followed by examples of amino acid substitutions not observed to attenuate hemolytic activity are: 17, Arg; 18, Asn; 33, Thr; 41, Gly; 45, Ala; 46, Thr; 63, Ser; 66, Tyr; 83, Ser; 101, Thr; 102, Gly; 127, Glu; 128, His; 153, Met; 172, Ala; 189, Arg; 239, Arg; 255, Gly; 257, Gly.

Please amend the paragraph bridging pages 13 and 14 as follows:

Random mutagenesis is one of the suitable techniques for introducing mutations into pneumolysin. Standard mutagenesis methods are suitable for use with this invention. In an embodiment, random PCR is performed in order to randomly incorporate nucleotide changes into the type 14 pneumolysin genome. The subsequent selection will identify desirable changes. This method is applicable with any isolated pneumolysin gene. Preferably, enough of the nucleic acid sequences is identified to enable production of oligonucleotide probes. Non-limiting examples of such pneumolysin genes are those

encoding for type 2 and 14 pneumolysin. The nucleotide sequence encoding type 14 is shown in Fig. 1A and Fig. 1B.

On page 16, lns. 15-28, please amend the second full paragraph as follows:

It is to be understood that the nucleotide sequences of this invention need not be limited to a single mutation within any given molecule encoding the modified pneumolysin polypeptides. Multiple mutations are also possible when they preserve the immunogenic character of native pneumolysin polypeptide (see Fig. 2A and Fig. 2B), while attenuating or eliminating one or more of its toxic characteristics. Multiple modifications may therefore be included in a single polypeptide molecule (see Fig. 4A and Fig. 4B). Multiple modifications may be useful because they may reduce the likelihood of reversion to the toxic native sequence. However, a preferred embodiment of this invention is single mutations in the nucleic acid sequence which result in single amino acid substitutions.

Please amend the paragraph bridging pages 23 and 24 as follows:

The modified pneumolysin polypeptides of this invention are polypeptides that are non-hemolytic or substantially non-hemolytic and still maintain at least one epitope that binds to antibody directed against the native polypeptide. Because such hemolytic activity is associated with the toxicity of pneumolysin, the modified pneumolysins would therefore also be expected to be less toxic than native pneumolysin. The modified pneumolysin polypeptides of this invention contain at least one mutation relative to *S. pneumoniae* type 14 wild-type pneumolysin (Fig. 3A and Fig. 3B), preferably among the first 257 amino acids beginning from the N-terminus. Modification of as few as one amino acid is required to result in modified pneumolysin polypeptides which have little or insignificant toxicity as determined by hemolytic assay. Thus, substitutions at any one, or more, of positions 61, 148 and 195 may result in polypeptides having reduced hemolytic activity. Preferred substitutions for amino acids 61, 148 and 195 are shown below in Table 1.

Under the section entitled "Bacterial Strains and Plasmids," please amend the paragraph bridging pages 44 and 45 as follows:

Streptococcus pneumoniae serotype 14 (ATCC, ~~Rockville, MD~~10801 University Boulevard, Manassas, VA 20110-2209) was used in this study for isolation of genomic DNA. *E. coli* strain DH5α (Life Technologies, Gaithersburg, MD) was used for initial cloning and production of plasmid DNA. *E. coli* strain BL21 (DE3)ΔompA, used for protein expression, was derived from BL21 (BE3) (Novagen) (see U.S. Patent No. 5,439,808 for details). *S. pneumoniae* was grown overnight in Todd-Hewitt (TH) broth at 37°C without shaking under 7.5% CO_2.. *E. coli* strains were grown in Luria-Bertani (LB) broth, supplemented with carbenicillin (50-100 μg/ml) or kanamycin (50 μg/ml) as needed. The plasmid vectors pUC-19 and/or pBluescript II SK+ (Stratagene) were used for cloning fragments to be sequenced and the plasmids pET-17b and pET-24a (Novagen) were used for cloning fragments to be expressed.

Under Example 2, please amend the paragraph bridging pages 46 and 47 as follows:

Genomic DNA was isolated from approximately 0.5 g *Streptococcus pneumoniae* serotype 14 using the method described above. This DNA served as the template for two pneumolysin-specific oligonucleotides in a standard PCR reaction. These oligonucleotides were designed to be complementary to the 5' and 3' flanking regions of the pneumolysin gene from *S. pneumoniae* serotype 2 and to contain XbaI restriction sites to facilitate the cloning of the fragment if desired. The sequence of the forward oligonucleotide was 5' AAC CTT GAT TGA TCT AGA TAA GGT ATT TAT GTT GG 3' (SEQ ID NO:5) and the reverse oligonucleotide had the sequence 5' TCT TTT TGT CTC TAG AAT TCT CCT CTC CTA GTC 3' (SEQ ID NO:6) . The PCR reaction conditions were as follows: 200 ng *S. pneumoniae* type 14 genomic DNA, the two oligonucleotide primers described above at 1 μM of each, 200 μM of each dNTP, PCR reaction buffer (10 mM Tris HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_3$, and 2.5 units of Taq polymerase, and QS. to 100 μl with dH_2O. This reaction mixture was then subjected to 25 cycles of 95C for 1 min, 50C for 2 min and 72C for 1.5 min. At the end of the cycling period, the reaction mixture was loaded on a 1.0% agarose gel and the material was electrophoresed for 2 h after which the band at 1.7 kb was removed and the DNA recovered using GeneClean® (Bio 101). This DNA was then digested with XbaI, repurified and ligated to XbaI-digested pUC-19 using T4 DNA ligase. The ligation mixture was used to transform competent *E. coli* DH5α. Recombinant plasmids were identified and sequenced; many were found to have a DNA sequence consistent with that of the gene encoding pneumolysin.

Under Example 3, please amend the paragraph bridging pages 47 and 48 as follows:

Plasmids capable of expressing the mature pneumolysin protein were constructed by amplifying DNA containing the full-length pneumolysin gene (pST20, pST85, or type 14 genomic DNA) with nested oligonucleotides designed to isolate the pneumolysin coding region. The forward oligonucleotide was designed to contain a NdeI site and would install a start codon at the 5' end of the coding region. This primer had the sequence 5' TAT TAG GAG GAG CAT ATG GCA AAT AAA GCA GTA AAT G 3' (SEQ ID NO:7). The reverse oligonucleotide was designed to contain an XhoI site and had the sequence 5' GGC CTC TTT TTG TCT CGA GCA TTC TCC TCT CCT AGT C 3' (SEQ ID NO:8). This strategy allowed the cloning of the fragment encoding mature pneumolysin into the NdeI and XhoI sites of either the pET-17b or pET-24a. Standard PCR was conducted using a template containing the entire pneumolysin gene (type 1, 2 & 14) and the two oligonucleotides described above. This PCR reaction yielded a 1.6 kb product when analyzed on a 1.0% agarose gel. The DNA obtained from the PCR reaction was gel purified and digested with the restriction enzyme NdeI and XhoI. The 1.6 kb product was again gel purified and ligated to NdeI- and XhoI- digested pET-17b or pET-24a using T4 DNA ligase. This ligation mixture was then used to transform competent *E. coli* DH5α. Colonies that contained the 1.6 kb insert were chosen for further analysis. The DNA from the DH5α clones was analyzed by restriction mapping and the cloning junctions of the chosen plasmids were sequences. After this analysis, the DNA obtained from the DH5α clones was used to transform *E. coli* BL21 (DE3)ΔompA. The transformed bacteria were selected on LB-agar containing 100 μg/ml of carbenicillin, or 50 μg/ml of kanamycin when using the pET-24a plasmid. Typically, several clones were screened for their ability to produce the mature pneumolysin protein.

Under Example 4, please amend the paragraph bridging pages 48 and 49, as follows:

A portion of the gene encoding pneumolysin comprising amino acid residues 1-257 was subjected to random mutagenesis using a modification of the technique as described. (Cadwell, R.C. and Joyce, G.F. (1994) *PCR Methods Appl.* **3**:pS136-40; Cadwell, R.C. and Joyce, G.F. (1992) *PCR Methods Appl.* **2**:28-33). An oligonucleotide complementary to the T7 promoter region of the pET-24a plasmid (See, Fig. 1A and Fig. 1B~~Figure 1a~~) with the sequence 5'ATT ACG CGA CTC ACT ATA GGG 3' (SEQ ID NO:9) and an oligonucleotide complementary to a region of the pneumolysin gene around 1250 bp (See, Fig. 1A and Fig. 1B~~Figure 1~~) with the sequence 5'ATT ACG AAC ATT CCC TTT AGG3' (SEQ ID NO:10) were used to define the region of the gene to be mutated. The random mutagenesis PCR reaction conditions were as follows: purified plasmid pNV-19.2 (100ng), the two oligonucleotide primers described above at 1 μM of each imbalance dNTP concentrations of 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, and 1 mM dTTP, PCR reaction buffer (19 mM Tris-HCl, 50 mM KCl, pH 8.3), 8.0 mM $MgCl_2$, 0.5 mM $MnCl_2$, 6 units Taq polymerase, and QS to 100 μl with dH_2O. This reaction mixture was then subjected to 40 cycles of 95°C for 1 minute, 40°C for 2 minutes, and 72°C for 3 minutes. After the PCR reaction, fragments were extracted with phenol/chloroform and ethanol precipitated. The fragment was then digested with NdeI and HindIII[[ ]], gel purified and ligated to pNV-19.2, digested with the same enzymes. The fragments were ligated and subsequently transformed into competent BL21 (DE3) *E.coli*.

Please amend the paragraph bridging pages 51 and 52 as follows:

Clones containing soluble pneumolysin are selected for the next step in the screening procedure, which consists of discarding the supernatant by aspiration, washing the pellet with TEN buffer twice, and solubilizing the pellet in 5 ml of 8 M urea prepared in TEN buffer. After sonicating for 2 min, the urea solution is quickly centrifuged to remove aggregates and added dropwise to 45 ml of refolding solution, under constant stirring at 4 °C. The refolding solution is then loaded onto a 2 ml DEAE-~~Sepharose~~SEPHAROSE-FF column, pre-equilibrated in Buffer A (25 mM Tris.HCl, pH 8.0). The column is washed with Buffer A and the bound protein is eluted with a gradient of 0 to 1 M NaCl. The properly refolded pneumolysin mutant should elute as a single peak between 13 and 20% Buffer B (25 mM Tris.HCl, 1 M NaCl, pH 8.0) similarly to what is observed for the wild-type. The protein peak is further analyzed by HPLC on a ~~Superose~~SUPEROSE 12 column and both elution time, aggregate/monomer ratio, and hemolytic activity are evaluated (see Table 4). The selected mutant(s) should present a single monomeric species with a Stokes radius comparable to the wild-type. Five clones (pNVJ1, pNVJ20, pNVJ22, pNVJ45, pNVJ56) with high yields of monomeric modified polypeptides were selected for further analysis including nucleic acid sequencing. The amino and nucleic acid substitutions of these clones are shown in Tables 5A and 6. Throughout the specification and claims, proteins are given the name of the vector that encodes them.

On page 58, please amend the section entitled "Table 7. Modified Pneumolysin Sequences" as follows:

MUTATION POSITION	AA #	Primer Sequence
443	148	Forward
		5'ggtcaggtcaataatgtcccagctagaaAgcagtatg 3' (SEQ ID NO:11)
	Met-Lys	Reverse
		5'gctgtgagccgtgatttttcatactgcTttctagctg 3' (SEQ ID NO:12)
583	195	Forward
		5'gcagattcagattgttaatGttaagcagatttattata 3' (SEQ ID NO:13)
	Phe-Ile	Reverse
		5'atctgcttaaCattaacaatctgaatctgcttttcgcc 3' (SEQ ID NO:14)
583	195	Forward
		5'cagattgttaatAttaagcagatttattatacagtcagc3' (SEQ ID NO:15)
	Phe-Val	Reverse
		5'aatctgcttaaTattaacaatctgaatctgcttttcgcc3' (SEQ ID NO:16)
181	61	Forward
		5'acaagtgatattCctgtaacagctaccaacgacagtcgc3' (SEQ ID NO:17)
	Ser-Pro	Reverse
		5'agctgttacagGaatatcacttgtatttgtcgacaagct3' (SEQ ID NO:18)

Please amend the paragraph on page 59, lns. 2-28 as follows:

Pneumolysin expressed in *E. coli* cells harboring the expression vector pNV19 was isolated from inclusion bodies by resuspending and lysing the cells in TEN buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA pH 8.0), with an air driven cell disrupter (Stansted Fluid Power Ltd.) under a pressure of 8,000 psi. The cell lysate was centrifuged at 13,000 rpm at 4°C for 20 minutes; both pellet and supernatant were saved for isolation of soluble and aggregated pneumolysin, respectively. The inclusion bodies were washed three times with TEN buffer and stored at -70°C Purification and subsequent refolding were achieved by solubilizing the inclusion bodies in an 8 M urea solution (freshly prepared in TEN buffer), followed by PEG-assisted refolding. Polypeptide solutions in 8 M urea (200 µg/ml) were diluted 10-fold by drop-wise addition to a refolding solution, consisting of 20 µM of PEG 8,000 in 25 mM Tris-HCl, pH 8.0, under constant stirring at 4°C. The sample was clarified and loaded into a DEAE-~~Sepharose~~SEPHAROSE Fast Flow ion exchange column (Pharmacia) equilibrated in 25mM Tris-HCl, pH 8.0. A gradient of 0-1 M NaCl was applied and pneumolysin containing fractions were identified by detection of hemolytic activity, as described below, and by SDS-PAGE. The purified fractions were concentrated by using an ~~Amicon~~AMICON concentrator and PM30 membrane. Aliquots of purified polypeptide were tested for hemolytic activity, and analyzed by SDS-PAGE and size exclusion chromatography, using a ~~Superose~~SUPEROSE 12 column.

Please amend the paragraph on page 63, lns. 5-24 as follows:

PnC type 14 polysaccharide (ATCC Lot #2016107) (390 mg) was dissolved in 16 ml of 0.5 N NaOH, and the solution was heated at 70°C for 3 hours. Following cooling of the solution, 1.93 ml of glacial acetic acid was added to bring the pH to 4. After addition of 3 ml of 5% (w/v) $NaNO_2$, the reaction mixture was kept stirring at 4°C for 2 hours. The sample was then diluted to 50 ml with deionized water and the pH was adjusted to 7 with 0.5 N

NaOH. Excess reagents were dialyzed out by diafiltration with DI water through a ~~Spectra/Por molecularporous~~SPECTRA/POR molecular porous membrane tubing (MWCOL:3,500), and the retentates freeze-dried. The deaminated type 14 polysaccharide was then molecular sieved on a ~~Superdex~~SUPERDEX G-200 (Pharmacia) column using PBS as eluent. Fractions eluting from the column with molecular weight between 5000 and 15,000 as determined by Chromatography/Multiangle Laser Light Scattering using a ~~Superose~~SUPEROSE 12 column (Pharmacia) were pooled and dialyzed against DI water through a ~~Spectra/Por molecularporous~~SPECTRA/POR molecular porous membrane tubing (MWCOL 3,500) and freeze-dried.

Please amend the paragraphs on page 64, ln. 3 through page 65, ln.3 as follows:

Modified pneumolysin polypeptides in 0.2 M phosphate buffer (pH 8) at a concentration of 5 mg/ml were mixed with 2.5 equivalents (by weight) of PnC 14 polysaccharide-fragment together with 2 equivalents (by weight) of recrystallized sodium cyanoborohydride . Reaction mixtures were incubated at 37°C for 24 hours. Conjugates were then purified from the free components by passage through a ~~Superdex~~SUPERDEX G200 (Pharmacia) column using PBS containing 0.01% thimerosal as an eluent. Fractions eluting from the column were monitored on a ~~Waters~~WATERS R403 differential refractometer and by UV spectroscopy at 280 nm. The fractions containing the conjugates were pooled, sterile-filtered through a 0.22 μm Millipore membrane and then stored at 4°C. Polypeptide and carbohydrate content were measured by the methods of Bradford and Dubois respectively. Polysaccharide content in the resulting conjugates were approximately 30%.

Tetanus toxoid conjugates for use as control, were also produced as described above and as follows: Tetanus toxoid (Serum Statens Institute) was first passed through a molecular sieve column (~~Superdex~~SUPERDEX G-200 Pharmacia) in order to obtain the monomer form of the toxoid. For conjugation, 12 mg of the monomer and 36 mg of the PnC 14 polysaccharide-fragments were dissolved in 600 μl of 0.2 M phosphate buffer pH 7.2. Recrystallized sodium cyanoborohydride (24 mg) was then added to the solution which was then incubated at 37°C for 3-days. The conjugate was purified as above. The conjugates had polysaccharide contents in the 25-30% range (see Table 10).

Please replace the paragraphs on page 66, lns. 4-28 with the following:

Micro titer plates (~~Nunc Polysorb~~NUN POLYSORB ELISA plates) were sensitized by adding 100 μl of type 14 polysaccharide-fragment (MW ca: 10,000)/HSA conjugate (2.5 μg/ml) in PBS. The plates were sealed and incubated at 37°C for 1 hour. The plates were washed with PBS containing 0.05% ~~Tween 20~~TWEEN 20 (PBS-T) and blocked with 0.5% (w/v) BSA in PBS for 1 hour at room temperature. The wells were then filled with 100 μl of serial two-fold dilutions in PBS-T plates, 100 μl of peroxidase labeled goat anti-mouse IgG (H+L) (Kirkegaard and Perry Laboratories), and then washed five times with PBS-T. Finally, 50 μl of TMB peroxidase substrate (Kirkegaard and Perry Laboratories) were added to each well, and following incubation of the plates for 10 minutes at room temperature, the reaction was stopped by the addition of 50 μl of 1 M H_3PO_4. The plates were read at 450 nm with a ~~Molecular Device Amex~~MOLECULAR DEVICE AMEX microplate reader using 650 nm as a reference wavelength.

Inhibition ELISA assay.

Microtiter plates (NUNC POLYSORP~~Polysorp~~) were coated with PLY (20 ng in 100 mL to each well) in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.4) for one hour at 37°C. After washing the plates with PBS + 0.05% ~~Tween 20~~TWEEN 20 (PBST), the plates were post-coated with 150 mL of PBS + 0.1% BSA, rewashed, and stored at 4°C until used.

Please amend the first paragraph on page 67, lns. 1-21 as follows:

Hyperimmune rabbit anti-PLY was diluted in PBST, added to the PLY coated plates, and incubated at room temperature for 1 h. After washing, 100 µL of goat anti-rabbit Ig-HRP conjugate (KPL) diluted in PBST[[ween]] according to the manufacturer's instructions were added to each well. The plate was incubated at room temperature for one hour and then washed again. 100µL of TMB microwell substrate (KPL) were added to each well. The reaction was stopped after 10 minutes by the addition of TMB one-component stop solution (KPL) and the $OD_{450\ nm}$ was immediately read. The dilution corresponding to 1/2 the maximum signal was chosen for the inhibition study. PLYD mutants as well as PLY as a control were diluted serially in three-fold ingrements in PBST containing the rabbit antiserum diluted such that the final mixture contained the dilution which gave half-maximal activity and applied immediately to the coated microtiter plates in duplicate. The plates were incubated at room temperature for one hour and processed. Inhibition was determined as percent of maximum signal achieved with dilute antiserum in the absence of any inhibitor.

Please amend the full paragraph on page 71, lns. 6-24 as follows:

The oxidized PSs were separately coupled to recombinant pneumolysoid mutant 207 in which amino acid Phe residue 195 was replaced by Ile. In brief, the oxidized PSs and the protein (5 mg/ml) in 0.2 M sodium phosphate buffer were combined at a PS/protein ratio of about 2.5:1 by weight at room temperature and sodium cyanoborohydride (2 equivalents by weight) was then added. The conjugation mixtures were incubated at 37°C for 2 days. After reduction of the residual aldehydes of the conjugated PS, with excess $NaBH_4$, the conjugates were purified from the reaction mixtures by passage through a column of ~~Superdex~~SUPERDEX 200 PG (Pharmacia) eluted with PBS containing 0.01% thimerosal as the preservative, except for the type 23 conjugate where the conjugate was loaded onto a Q ~~Sepharose~~SEPHAROSE Fast Flow column, and eluted with 10 mM Tris-HCl, pH 7.5 using a gradient of 0.5 M NaCl. Fractions corresponding to the conjugates were pooled and analyzed for protein and carbohydrate content as described in [[e]]Example 8 (see Table 12).